Transcriptome analysis of ovary culture-induced embryogenesis in cucumber (*Cucumis sativus* L.)

Ying Deng[1,2†], Wenyuan Fu[2†], Bing Tang[2,] , Lian Tao[2], Lu Zhang[1], Xia Zhou[1] Qingqing wang[2], Ji Li[1] and Jinfeng Chen [1*]

[1]State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China.

[2] Institute of Horticulture, Guizhou Academy of Agricultural Sciences, Guiyang55006 , China

[†] These authors contributed equally to this work.

*Please address all correspondence to: J F Chen (jfchen@njau.edu.cn)

Dr. Jin-Feng Chen

Professor

State Key Laboratory of Crop Genetics and Germplasm Enhancement,

Nanjing Agricultural University

Nanjing 210095, China

Fax: +86-025-84396279

E-mail: jfchen@njau.edu.cn.

Field Code Changed

Email addresses:

Ying Deng:87928883@qq.com

Wenyuan Fu: 742650745@qq.com

Bing Tang: 447260105@qq.com

Lian Tao: 499892784@qq.com

Lu Zhang: 593356478@qq.com

Xia Zhou: 1696546140@qq.com

Qingqing wang:1943922959@qq.com

Field Code Changed

Ji Li: liji1981@njau.edu.cn

Field Code Changed

Jinfeng Chen:jfchen@njau.edu.cn

Abstract

Background. Ovary culture has been a useful way to generate double haploid (DH) plants in cucumber (*Cucumis sativus* L.). However, the rate of embryo induction and the ability for induced embryos to grow into normal embryo are quite low. Moreover, the mechanism of cucumber embryogenesis remains ambiguous. In this study, the molecular basis for cucumber embryogenesis was explored to set up basis for a more efficient ovary culture method. Differentially expressed genes during the process of embryogenesis, including the early stage of embryo formation, embryo maturation and shoot formation, were investigated using transcriptome sequencing.

Methods. Based on cytological and morphological observations of cucumber ovary culture, ovary culture can be divided into three stages: early embryo development (T0), embryo maturation (from pro-embryos to cotyledon embryos, T1, T2, T3 and T4) and the shoot formation stage (T5). Six key time points were T0 (the ovules were cultured for 0 d), T1 (the ovules were cultured for 2 d), T2 (the embryos were cultured for 10 d), T3 (the embryos were cultured for 20 d), T4 (the embryos were cultured for 30 d), and T5 (the shoots after culture for 60 d), which were selected for transcriptome sequencing and analysis.

Results. Characteristics of developmental transformation of cucumber during embryogenesis and plant regeneration have been observed by cytology and morphology. Analysis of differentially expressed genes at developmental transition points by transcriptome sequencing. In early embryo development, the cells expanded， which is the symbol for gametophytes to switch to sporophyte development pathway. In early embryogenesis stage, RNA-seq revealed 3468 up-regulated genes compared to fresh unpollinated, including hormone signal transduction genes, hormone response genes and stress-induced genes. The reported embryogenesis-related genes *BBM, HSP90* and *AGL* were also actively expressed during this stage.

In embryo maturation stage, from cell division to cotyledon-embryo formation, 480 genes that function in protein complex binding, microtubule binding, tetrapyrrole binding, tubulin binding and other microtubule activities were continuously up-regulated during T1, T2, T3 and T4 timepoints, indicating that the cytoskeleton structure was continuously being built and maintained by the action of microtubule-binding proteins and enzyme modification. At the shoot formation stage, 1383 genes were up-regulated, which were mainly enriched in phenylpropanoid biosynthesis, plant hormone signal transduction, phenylalanine metabolism, and starch and sucrose metabolism. These up-regulated genes included 6 transcription factors that contained a B3 domain, 9 genes in the AP2/ERF family and 2 genes encoded *WUS* homologous domain proteins.

Conclusions. These findings offer a valuable framework for generating hypotheses regarding the transcriptional regulatory mechanism underlying embryogenesis during cucumber ovary culture.

Key words: Cucumber; Ovary culture; Embryogenesis; Transcriptome; Differentially expressed genes

Introduction

Gametophyte culture involves stress-induced reprogramming of male or female gametophytes to develop into embryo-like structures, which can be directly regenerated into completely homozygous, doubled haploid (DH) plants. The study of male gametophyte (microspore) embryogenesis began in the 1960s (Guha et al., 1964). Microspore embryogenesis in vitro can be easily monitored and provides a convenient experimental platform for large-scale physiological and biochemical analyses (Malik et al., 2007). However, the study of gynogenesis is more complicated; the embryo sac is small and embedded in surrounding tissues, making early embryos difficult to observe and isolate. For plants of recalcitrant to androgenesis, e.g. cucumber or self-incompatibility, male-sterile and dioecious plants, gynogenesis is worthwhile (Pazuki et al. 2018). In vitro gynogenesis is mainly used in the *Cucurbitaceae*, and previous studies have revealed that this technique is still imperfect and that the embryogenesis rate is low (Metwally et al., 1998; Gémes et al., 2002; Du et al., 2003; Shalaby, 2007; Diao et al., 2009; Li et al., 2013; Plapung et al., 2014; Tantasawat, 2015). The study of the mechanism of embryogenesis has therefore been delayed, and the understanding of the mechanisms underlying gynogenesis is extremely limited.

In general, induction of embryogenesis using in vitro culture is a stress-induced phenomenon. Micropores are cultured and develop into embryos in vitro, and stress treatments involving cold or heat shock and hormones are used as trigger factors to induce gametocytes to follow the sporophyte development pathway (Touraev et al., 1997). The genes associated with reprogramming phase and the early stage of embryogenesis were successively characterized, such as *AGL15-related* (*AGAMOUS-LIKE15-related*) genes and the *AGL15* (*AGAMOUS-LIKE15*) gene, the *BBM* (*BABY BOOM*) gene and the *HSP* (*HEAT SHOCK PROTEIN*) genes (Perry et al.,1999; Boutilier et al., 2002; Maraschin, 2005). More recently, functional genomics has made it possible to identify more genes associated with different stages of microspore embryogenesis. In *Brassica napus*, the expression of embryogenesis-related genes such as *BBM* , *LEC1* (*LEAFY COTYLEDON1*) and *LEC2* (*LEAFY COTYLEDON2*) were detected after culture under heat stress at 32 °C for three days; after incubation at 24 °C, embryo-specific expressed genes, such as *LEC1*, *LEC2* were detected (Malik et al., 2007). The expression of genes associated with metabolism, chromosome remodeling, transcription, and translation signaling was up-regulated during the stress

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treatment stage in tobacco (Hosp et al., 2007). Similarly, genes related to metabolism, cell walls and the cell membrane, cell tissue control, cell communication and signal transduction were detected in the early stage of rape embryogenesis (Rosa Angélica et al., 2013). High levels of *BBM* and *LEC* gene expression have been confirmed in the early embryonic development of sweet pepper anther cultures (Irikova et al., 2012). The transition from microspores to developing embryos is mainly manifested in the induction of transcription factor genes that play an important role in early embryogenesis, many genes involved in hormone biosynthesis and plant hormone signal transduction in addition to genes involved in secondary metabolism (Bélanger et al., 2018). Studies have shown that differentially expressed genes can be detected by gene chips in *in vitro* gynogenesis of cucumber in the early stage and have suggested that phenylalanine metabolism and phenylalanine synthesis may play an important role in the early development of cucumber in vitro (Zhang, 2013).

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Cucumber (*Cucumis sativus* L.) is the fourth most important vegetable worldwide (Lv et al., 2012), accounting for more than 2.2 million hectares and a total production of approximately 83 million tons in 2017 (http://www.fao.org/faostat). Cucumber is regarded as one of the oldest vegetable crops and has been domesticated in China for probably 2000 years (Golabadi et al., 2012). To meet the needs of production, breeders are constantly looking for valuable germplasm resources with valuable characteristics, especially resistance to disease and environmental stresses such as cold, drought, or salt stresses (Wang et al., 2018). The collection of extensive germplasm resources for variety improvements can greatly facilitate these breeding efforts. However, cucumber is a cross-pollinated plant with obvious heterosis. Therefore, almost all the cucumber varieties in production are hybrids; leading to slow breeding processes. In the breeding of parents, it takes 6-8 years of artificial self-breeding to develop a stable inbred line. To accelerate the purification of cucumber parents and improve breeding efficiency, haploid gametophyte cultures can be used to induce embryoids, and homozygous double haploids can be obtained in 1-2 years. DH technology is a powerful tool to speed up plant breeding. However, additional research is needed to improve our understanding of the genes or the roles of genes involved in embryogenesis and the mechanism of haploid induction in embryo sacs (Chen et al., 2011).

This study was based on the highly efficient in vitro ovary culture technology system of cucumber. The process from acquiring embryogenic potential to plant regeneration was divided into three stages: early embryo development, embryo maturation (from pro-embryos to cotyledon embryos) and shoot formation. We evaluated the embryo morphology and transcriptomes at different developmental stages during embryogenesis in cucumbers via ovary culture. The metabolism and biological process of embryogenesis in the gynogenesis of cucumber are discussed, and several key genes regulating embryogenesis were identified.

Materials and methods

Plant materials

'SG033' (F_1 cultivar of Kunming Huaxing seed Industry Co., Ltd., Kunming, Yunnan) was used for ovary culture; this material had a high embryo generation rate after a large number of screenings. Cucumber plants from southern China were cultivated and stored in our lab. The protocol for plant growth was slightly adapted from (Li et al., 2014). Specifically, the plants were grown in a 12-hour photoperiod greenhouse with an average daily air temperature of 25/15 °C (day/night), a relative humidity of 85% and a photosynthetic luminous flux density of 500 μmol·m^{-2}·s^{-1} at the Horticulture Institute of Guizhou Academy of Agricultural Sciences, Guizhou, China. Ovaries were collected from 'SG033' for one month after the first anthesis flower appeared.

Culture medium composition

MS medium was used as a basal medium; it was supplemented with 0.06 mg·L^{-1} TDZ (thidiazuron, solarbio) and 3% (w/v) sucrose and was solidified with 7% (w/v) agar. The pH of the medium was adjusted to 5.9 before autoclaving at 116 °C and 1.1 kg/cm2 for 30 min.

Ovary culture

Ovaries were harvested at 6 h before anthesis, and treated for 24 h in a refrigerator at 4 °C. Their papilloma was removed. The ovaries were then sterilized in 75% ethanol for 30-60 s and rinsed in sterile distilled water 3 times, followed by soaking in a 0.5% sodium hypochlorite for 20 min and then rinsing in sterile distilled water 3 times. The ovaries were cut into small round slices of 2 mm under sterile conditions and then placed on 30 mL of solid media in cylindrical flasks. All materials were treated with high temperature of 33 ℃ for 2 d, after which recovery growth was allowed until shoot formation occurred at 25 °C under a 16/8 h (light/dark) photoperiod with a 4000 lux light intensity.

Observation

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The observation of embryos development was performed according to the method published by our team (Deng et al., 2020). Specifically, SG033 explants (ovarian fragments) were collected before and after culture and resin sections were performed to observe the development of early embryos. Fifty explants were collected respectively from 0 d and 2 d and were fixed in FAA (formalin:glacial acetic acid:70% ethanol in a ratio of 1:1:18) at room temperature for 48 h, then dehydrated with a graded ethanol series and embedded in the spurr resin. Nine-micrometer-thick sections were stained with safranine and fast green, and observed using a biological microscope (Leica DM2500, Wetzlar, Germany). After 10 d of culture, the embryonic morphology was evaluated by stereomicroscope (Leica M165 C, Wetzlar, Germany).

RNA isolation

The materials were harvested separately at the following time points: T0 (the ovules were cultured for 0 d, i.e. fresh unpollinated ovaries), T1 (the ovules were cultured for 2 d), T2 (the embryos were cultured for 10 d), T3 (the embryos were cultured for 20 d), T4 (the embryos were cultured for 30 d), and T5 (the shoots after culture for 60 d). Heart chamber was cut free-hand in T0 and T1 samples under stereomicroscope respectively, embryos of the T2-T4 were dissected from the undifferentiated tissue, and shoots were selected directly in the T5. 200 mg for each sample were used as starting material for the RNAseq and qPCR experiments.

These materials were stored at -80 °C for subsequent RNA extraction, and the remaining materials were maintained in culture to observe plantlet regeneration. Furthermore, all experimental procedures, such as culture medium replacement and sample collection, were performed under similar conditions to minimize possible circadian effects. Ovules at T0 and T1 were extracted by hand under a stereoscopic microscope, and embryo-like structures with integuments were selected directly at the time points from T2 to T5.

As described above, samples were collected from six time points of ovary culture for RNA-seq analysis. For each sample, the ovules or embryo-like structures were immersed in liquid nitrogen in a mortar and ground into powder (three biological replicates per sample). Total RNA was isolated using TRIzol (Invitrogen), and then DNase I (Fermentas) digestion was performed for 30 min at 25 °C to remove DNA, according to the manufacturer's instructions. The integrity and quality of the total RNA were checked using a NanoDrop 1000 spectrophotometer and formaldehyde-agarose gel electrophoresis. RNA was used only when the OD_{260}: OD_{280} ratio was above 1.8.

Library construction and sequencing

The library construction and sequencing were performed as previously published (Liu et al., 2014). Specifically, the library was constructed with 3 micrograms of total

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RNA and enriched with magnetic beads coated with oligosaccharide (DT). After adding fragment buffer, the mRNA was converted to short fragments (200-300 bp). Next, mRNA fragments were used as templates to synthesize cDNA. The double stranded cDNA was purified with the kit of QiaQuick PCR, washed with EB buffer solution, repaired at the end and added with single adenine (A) nucleotide. Finally, the sequencing adapters were connected to the fragments. The fragments were purified by agarose gel electrophoresis and amplified by PCR. The library products were then transferred to Illumina HiSeq™ 2000 for sequencing analysis. It is important that the base calling was used to convert the original image data into sequences in order to obtain clean reads before further analysis.

Transcriptome analysis

Analysis of transcriptomes was performed with Illumina Hiseqtm2000. Using Illumina GA Pipeline (v1.6) software we removed more than 10% unknown base and low-quality base readings from the raw sequencing data. Using TopHat2 (http://tophat.cbcb.umd.edu/) software we aligned the filtered reads with the Chinese long v2 genome of Cucumber. Reference genome and gene database information are available from the public website: http://cmb.bnu.edu.cn/Cucumis_ sativus_ V20 / (Huang et al., 2009). Differential expression analysis was done using DESseq (version 1.18.0). Benjamini and Hochberg's methods were used to control false discovery rate and the obtained *P* values were adjusted. Genes with an adjusted *P* value of <0.05 and an absolute value of |log2fold change≥1| according to DESeq were considered differentially expressed. GO annotation was implemented by Blast2GO software (GO association was performed through a BLASTX search of the NCBI NR database). Then, the GO enrichment analysis of differentially expressed genes (DEGS) was carried out by the BiNGO plugin.

Overrepresented GO terms were recognized using a hypergeometric test after Benjamini-Hochberg FDR correction with a significance threshold of 0.05. The KOBAS (2.0) software was used to carry out the KEGG enrichment analysis of the differentially expressed genes (Mao et al., 2005). The "*p* value ≤0.05" was used as the threshold to judge the significant enrichment pathway of differentially expressed genes.

Real-time quantitative PCR

The validation of the RNA-seq technique was performed by quantitative RT-PCR through monitoring the expression levels of ten selected transcripts. The total RNA of the RNA-seq samples was treated with DNase I enzyme, and then transformed into single-strand cDNA by using GoScriptTM Reverse Transcription System (Promega) on the basis of the manufacturer's protocol. According to the cDNA sequence in table S1, primers 5.0 were used to design specific gene primers. Actin is used as an internal

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control to normalize minor differences in the number of templates. Quantitative real-time PCR was performed with GoTaq qPCR Master Mix (Promega) in a Bio-Rad iQ1 real-time PCR system (Bio-Rad). The expression ratio was calculated using the $2^{-\Delta\Delta CT}$ method showing the average of three technical replicates.

Results

Morphological and cytological characterization of *in vitro*-induced embryogenesis in cucumber

The early embryogenesis stage, embryo maturation stage and shoot formation stage were observed to discern the cytological changes during embryogenesis by ovary culture in cucumber. Ovaries at 6 h before anthesis were collected and pretreated at 4 ºC. The ovaries were sterilized, sliced, inoculated into the medium, incubated for 2 d at 33 ºC in the dark and then transferred to 25 ºC until plants formed. Interestingly, based on the morphological and cytological observations, critical developmental transitions in embryos were discovered at 2 d (T1), 10 d (T2), 20 d (T3), 30 d (T4) and 60 d (T5) of culture. Fresh unpollinated ovaries before culture were named T0 (Fig. 1). At T0, fresh unpollinated ovaries were selected, in which the ovules were obvious (Fig. 1a) and the embryo sacs were mature (Fig. 1g). At T1, heat shock stress induced the embryogenesis of cucumber (Fig. 1b). The most obvious sign that referred to the development of the embryo was the enlargement of the cells in the embryo sac (Fig. 1h). The enlargement of microspore has been correlated with embryogenic potential acquisition during induction of androgenesis in many crop species (Hoekstra et al., 1992; Maraschin, 2005). After culturing for 2 d, 4 d and 6 d, the expanded cells formed two (Fig. 1i), four (Fig. 1j) and multiple cell structures (Fig. 1k), respectively. At T2 (10 d), the ovules had successfully protruded from the surrounding tissues, and a pro-embryo had formed (Fig. 1c). At T3 (20 d), the total number of embryos had increased in one ovary slice (Fig. 1d). From T3 to T4 (culture for 30 d), the whole embryo had formed, including globular embryos, heart-shaped embryos, torpedo-shaped embryos and cotyledon-shaped embryos (Fig. 1 l). At the same time, the color of the embryonic cells appeared green, indicating that the development of plants from the embryo would begin (Fig. 1e). At T5 (60 d after culture), shoots had formed (Fig. 1f).

At T1, ovule enlargement and cell enlargement could be clearly observed, suggesting that the transformation of the development pathway from the gametophyte

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to the sporophyte was induced by stress and that the stage between T0 and T1 was the key to inducing embryogenesis. After the switching of cell development, embryogenic potential was acquired. Mitosis then occurred continuously to form pro-embryos, globular embryos, heart-shaped embryos, torpedo-shaped embryos and cotyledon-shaped embryos, similar to zygote development, and this process continued until T4. After 60 d of culture, the mature embryos developed into shoots. Therefore, we divided the six time points of ovary culture into three stages: T0 to T1 (early embryogenesis stage), T1 to T4 (embryo maturation stage), and T4 to T5 (shoot formation stage). Transcriptome data were generated via Illumina II HiSeq™ 2000 sequencing, and the statistics of RNA-seq alignment were shown in Table S2.

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Validation of differentially expressed genes

Validation of the Illumina sequencing data and the expression patterns of the DEGs revealed by RNA-seq was performed to examine the expression patterns of 10 DEGs, including 7 genes involved in plant hormone signal transduction and 3 plant-pathogen interaction genes. The results showed that the relative expression levels revealed by RNA-seq and qRT-PCR were closely correlated (Pearson's r = 0.53, 0.77, 0.57, 0.74, 0.79, 0.69, 0.81, 0.81, 0.86, 0.62, 0.81). The fold changes in the qRT-PCR analysis were different from those in the RNA-seq analysis, which might be due to the difference in sensitivity between the qRT-PCR analysis and the RNA-seq technique. However, the qRT-PCR analysis showed that the up- and down-regulation trends of the differential gene expression were consistent with those of the RNA-seq analysis (Fig. 2).

Expression of embryogenesis-related genes in cucumber ovary culture

At the early stage of embryogenesis, after heat shock stress, ovule enlargement was obvious (Fig. 1b). Cytological observations also showed that one of the cells in the embryo sac expanded (Fig. 1h), suggesting acquisition of embryogenic potential.

The differentially expressed genes at this stage (T0 vs T1) were identified as comprising 3468 up-regulated genes and 3065 down-regulated genes (Fig. 3). We performed a GO enrichment analysis of these genes, and the results showed that the proteins encoded by these genes were assigned to 4 biological processes, 3 molecular functions and 9 cellular components (Table S3). The majority of DEGs were involved in the ‘single-organism process’ (GO:0044699) and the ‘oxidation-reduction process’ (GO:0055114). They were mainly distributed among the terms ‘membrane’

(GO:0016020), ‘membrane part’ (GO:0044425), ‘intrinsic component of membrane’ (GO:0031224) and ‘integral component of membrane’ (GO:0016021), having good molecular function in oxidoreductase activity (GO:0016491). Among the DEGs, the expression of related genes involved in the functional classification might play an important role in early embryogenesis.

To compare and summarize the results of this stage, we performed a pathway analysis to identify potential target genes. Based on the KEGG database, the pathway enrichment analysis of these genes revealed significant involvement in 8 distinct pathways (Fig. 4): pathways involving plant hormone signal transduction, phenylpropanoid biosynthesis, plant-pathogen interaction, glutathione metabolism, cysteine and methionine metabolism, drug metabolism-cytochrome P450, phenylalanine metabolism and metabolism of xenobiotics by cytochrome P450.

Plant hormone signal transduction genes

The DEGs were significantly enriched in the plant hormone signal transduction pathway (Ko04075), and a large number of genes, related to cytokinin and ethylene signalling pathways, encoding histidine phosphotransferase protein (*Csa2M373410.1*, *Csa6M067360.1*, *Csa7M452370.1*), response regulators (RR) (*Csa1M006300.1*, *Csa3M822100.1*, *Csa4M436980.1*, *Csa5M223020.1*, *Csa5M434550.1*, *Csa5M603910.1*, *Csa5M623800.1*, *Csa6M383530.1*), EIN3 binding (F-box) proteins (EBF) (*CsaUNG009930*) and ethylene-responsive protein transcription factors (ERF) (*Csa3M389850.1*) were significantly up-regulated after heat treatment. Moreover, the partially up-regulated DEGs encoding auxin influx carriers, auxin-responsive proteins, SAUR family proteins, abscisic acid receptor proteins, serine/threonine-protein kinases, ABA-responsive element binding factors, and brassinosteroid signal-responsive protein kinases were significantly enriched in 8 pathways, some of which involved tryptophan metabolism as well as zeatin, carotene, and brassinosteroid biosynthesis, suggesting that hormone production and hormone signal transduction were closely related to stress-induced embryogenesis. The up-regulated genes encoding major enzymes and receptor proteins involved in the plant hormone signal transduction pathway were expressed during the whole stage of ovary culture (Fig. 5). Most of the genes were expressed dynamically after their up-regulated expression during embryogenesis. Two genes (*Csa6M147590.1*, *Csa3M389850.1*) were up-regulated only at the stage of embryo initiation; both showed almost no expression in the later stages. *Csa6M147590.1* and *Csa3M389850.1* encode auxin-induced proteins

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and ethylene-responsive transcription factors, respectively, indicating that they might be closely related to embryogenesis.

Stress response proteins related to plant defense mechanisms

DEGs were significantly enriched in the plant-pathogen interaction pathway, which is related to plant defense mechanisms and complex physiological responses to heat shock-induced embryogenesis. In this regulatory pathway, the expression of *Pti1* (*Pto-interacting protein 1*, *Csa7M420160.1*), *HSP90* (*Heat shock protein 90*, *Csa3M183950.1*), *EDS1* (*Enhanced disease susceptibility 1*, *Csa1M006320.1*) and other related genes were up-regulated. *HSP90* was up-regulated under heat stress. This gene had been highly conserved during evolution and has plant defense functions.

Embryogenesis-related transcription factors

Transcription regulation, through transcription factors, is a key step in the response of plants to stress. In the embryogenesis stage, after heat shock stress, the expression of a total of 32 transcription factors was up-regulated. Among them, the *AP2/ERF*, *WRKY*, *bHLH* (*basic helix loop helix*), *MYB*, *GATA*, *HSF* (*heat stress factor*) and *NAC* (*NAM* / *ATAF* / *CUC*) families had more transcription factors. There were 26 transcription factors up-regulated by AP2/ERF family, which played an important role in ethylene response, brassinolide response, biological and abiotic stress responses. Two of them, *BBM* (*CSA3M827310.1*, *CSA3M827320.1*), were up-regulated. Secondly, 19 transcription factors were up-regulated in *WRKY* family, which is involved in the physiological process of embryo development, metabolism, environmental stress and defense response to pathogens. *bHLH* family plays an active role in regulating environmental stress. *MYB* family controls cell cycle by controlling different stages of cell division, and then promoted embryogenesis. After heat shock stress, four *HSF* (*Csa2M356690.1*, *Csa6M517310.1*, *Csa1M629180.1*, *Csa3M822450.1*) were specifically expressed, which can activate the *HSP90* gene responding to heat stress. The up-regulated expression of these transcription factors might be involved in embryogenesis and actively regulate biological and abiotic stress processes.

Response of genes involved in microtubule organization in cucumber ovary culture

In the stage of embryo maturation, the gametophyte development pathway switched to the sporophyte development pathway, in which the cells enlarged and initiated mitosis,

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forming 2-cell, 4-cell and multicellular pro-embryos after heat stress (Fig. 1h~k); afterward, globular embryos, heart-shaped embryos, torpedo-shaped embryos and cotyledon embryos were formed (Fig. 1l). The DEGs analyses of T1 vs T2, T2 vs T3 and T3 vs T4 revealed 480 continuously expressed genes. Additionally, the numbers of uniquely expressed genes between T1 and T2, T2 and T3 and T3 and T4 were 2236, 371 and 1558, respectively (Fig. 6).

GO categories of the 480 continuously expressed genes were significantly assigned to 2 biological processes, including cell movement or subcellular component (GO:0006928) and microtubule-based movement (GO:0007018), which were mainly associated with microtubules (GO:0005874), supramolecular fibers (GO:0099512) and polymeric cytoskeletal fibers (GO:0099513). Subsequently, proteins encoded by these genes were classified into 11 functional categories, including protein complex binding (GO:0032403), microtubule binding (GO:0008017), tetrapyrrole binding (GO:0046906), tubulin binding (GO:0015631), microtubule motor activity (GO:0003777), motor activity (GO:0003774), macromolecular complex binding (GO:0044877), heme binding (GO:0020037), oxidoreductase activity (GO:0016491), iron ion binding (GO:0005506) and oxidoreductase activity (GO:0016491), acting on paired donors or the molecular incorporation of oxygen (GO:0016705) (Table S4). These findings indicated that, during certain stages, the cytoskeleton structure was continuously built and maintained by the action of microtubule binding proteins and enzyme modifications, providing the basis for positioning the various organelles and implementation functions, which ensured the orderly activities in various cells in time and space.

To determine the involvement of these differentially expressed genes in embryo development, we performed a pathway analysis to identify the potential target genes, and 17 significant pathways were obtained by mapping to the KEGG database (Fig. 7): protein processing in the endoplasmic reticulum, phenylpropanoid biosynthesis, photosynthesis-antenna proteins, limonene and pinene degradation, meiosis-yeast, the estrogen signaling pathway, cell cycle, cell cycle-yeast, diterpenoid biosynthesis, chloroalkane and chloroalkene degradation, carotenoid biosynthesis, progesterone-mediated oocyte maturation, glycerolipid metabolism, histidine metabolism, fatty acid degradation, antigen processing and presentation, and ascorbate and aldarate metabolism. Among them, the pathways of protein processing in the endoplasmic reticulum and phenylpropanoid biosynthesis were the most significant.

Expression of main oxidation-reduction and metabolic process-related genes in cucumber ovary culture

In the stage of shoot formation, the embryos further differentiated into shoots (Fig. 1f). In total, 3320 genes were differentially expressed between T4 and T5, among which 1383 genes were up-regulated and 1837 were down-regulated in T5 (Fig. 3). Next, we performed an enrichment analysis of the genes using Gene Ontology (GO), which revealed 18 biological processes, 15 molecular functions and 5 cellular components (Table S5). The terms annotated under the biological process category mainly included oxidation-reduction processes (GO: 0055114), the regulation of primary metabolic processes (GO: 0080090), the regulation of cellular metabolic processes (GO: 0031323), and the regulation of macromolecule metabolic processes (GO: 0060255). The molecular function category mainly included the terms oxidoreductase activity (GO: 0016491) and DNA binding (GO: 0003677). The cellular component category mainly included extracellular region (GO: 0005576). The development of the ovary culture was mainly focused on the process of oxidation-reduction and metabolism.

The DEGs were subsequently annotated using the KEGG database to identify pathway enrichments. A variety of pathways were found to be significantly enriched (Fig. 8), including those involved in phenylpropanoid biosynthesis; plant hormone signal transduction; stilbenoid, diarylheptanoid and gingerol biosynthesis; metabolism of xenobiotics by cytochrome P450; drug metabolism-cytochrome P450; flavonoid biosynthesis; phenylalanine metabolism; starch and sucrose metabolism; and zeatin biosynthesis. Most of the differentially expressed genes were enriched in phenylpropanoid biosynthesis, plant hormone signal transduction, phenylalanine metabolism, and starch and sucrose metabolism. Pathways involving phenylpropanoid biosynthesis and phenylalanine were important for metabolizing secondary metabolites in plants and were closely related to cell differentiation and pigmentation in plant development. In the plant hormone signal transduction pathway, the expression of an auxin response factor, the gibberellin receptor *GID1*, ethylene-responsive transcription factor 1 (*ERF1*) and cyclin D3 were up-regulated, promoting plant development and maturation, cell enlargement and division as well as promoting the development of stems. In addition, the DEGs involved in starch and sugar metabolism were also significantly enriched and mainly encoding 3-βD glucosidase, T6Ps, GlgB, endoglucanase and alpha-trehalase, which satisfied the requirements of embryo growth and development.

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Shoot formation-related transcription factors

In the stage of shoot formation, 62 transcription factors were up-regulated, belonging to 23 transcription factor families, and the up-regulation ratio was between 2.03 and 214.13. The most up-regulated gene was the *TGA* (*TCACG motif-binding factor*) transcription factor (*CSA4M625020.1*) of the *bZIP* (basic leucine zipper) family, which can specifically bind to the activation sequence with *TGACG* as the core to regulate the transcription level of target genes and play an important role in the defense response against biological and abiotic stress and organ development. The *AP2 / EFR, bHLH, MYB, WRKY, ZIP* and *WOX* (*WUSCHEL-related homeobox*) were the families with a large number of transcription factors, which might be important molecular clues for shoot formation.

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Discussion

Many previous experiments have shown that early embryogenesis of microspore cultures can be divided into three main phases: acquisition of embryogenic potential, initiation of cell division, and pattern formation (Maraschin, 2005). In the present study, the whole process was investigated from acquisition of embryogenic potential to plant regeneration. We divided the ovary culture into three stages. In the early stage of embryogenesis, the acquisition of embryogenic potential by stress (e.g., low or high temperature and hormone induction) was observed together with the repression of gametophytic development, leading to the dedifferentiation of cells. In the embryo maturation stage, cell divisions gave rise to the formation of pro-embryos (cell clumps), globular embryos, heart-shaped embryos, torpedo-shaped embryos and cotyledon embryos (mature embryos). In the stage of shoot formation, mature embryos developed into shoots. There are active molecular events that regulate embryo development at different stages of development.

Embryogenesis-related genes are expressed in cucumber ovary culture

The embryogenic ability and transformation of somatic cells were regulated by various plant hormones, such as auxin, abscisic acid, cytokinin and ethylene (Ikeda-Iwai et al., 2003). Moreover, the synthesis of auxin and ethylene increased significantly after ovary pollination and fertilization (Mól et al., 2004). In normal growth, mitotic asymmetry of fertilized eggs was found in the gametophyte development pathway. Previous work using molecular and genetic approaches has identified auxin as a critical signal for the proper development of the asymmetric structure of the female gametophyte in Arabidopsis (Pagnussat et al., 2009). In *in vitro* culture, a certain stress condition is needed to block the development direction of the original gametophyte, followed by turning into the direction of the sporophyte and carrying on the symmetry splitting to eventually lead to embryogenesis (Fan et al.,1988). In ovary culture of cucumber, heat shock pretreatment, silver nitrate, genotype and hormone combination factors could play key roles in embryo and callus production independently and simultaneously (Golabadi et al., 2017). In the ovary culture of linseed, cultivar, combination of growth regulators, type of carbohydrates and their interaction significantly influenced callus induction and shoot formation frequency, and a relatively high shoot regeneration frequency was obtained when the medium was supplemented with TDZ and NAA (Aušra et al., 2017). These results implied that the expression of specific genes in embryogenesis was activated by exogenous hormone regulation, which laid the foundation for DNA replication in the cell division stage.

In our study, a large number of DEGs was enriched in the plant hormone signal transduction pathway during the stress process of embryogenesis. The pathway annotation obtained by KEGG analysis showed that phytohormones such as cytokinin and ethylene were significantly up-regulated in multiple biosynthesis and metabolic pathways, while up-regulated and down-regulated genes related to auxin and abscisic acid both existed. Generally, high-level endogenous cytokinin and low-level endogenous ethylene are beneficial for the acquisition of embryogenic ability. Embryogenesis is the comprehensive performance of interactions among different hormones, and all kinds of endogenous hormones show dynamic changes. The up-regulated expression of the main enzymes and receptor proteins in the plant hormone signal transduction pathway could promote embryogenesis, indicating that the high expression of related genes may play important roles in the process of switching from the gametophyte to the sporophyte development pathway. In this pathway, we found two genes related to hormone regulation that were highly expressed at the stage of early embryo development (*Csa6M147590.1* and *Csa3M389850.1*); moreover, the functional categories of their specific functions and the location of the regulatory pathways were identified, thus laying an important theoretical basis for the elucidation of the mechanism.

Heat shock protein 90 (HSP90) is an evolutionarily conserved molecular chaperone induced by abiotic stress. HSP90 plays an important role in cell cycle control,

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genomic silencing, protein transduction and signal transduction (Zhang et al, 2013). The HSP90 chaperone is involved in maintaining phenotypic plasticity and developmental stability (Hahn et al.,2011, Sangster et al., 2005). Previous studies have shown that *HSP* genes were specifically expressed in the spore stress-induction process of many crop species (Zarsky et al., 1995; Binarova et al., 1997; Sabehat et al., 1998). *HSPs* had been described as genes associated with the reprogramming phase and the early stages of embryogenesis (Maraschin, 2005). After heat shock stress treatment, we found that 26 up-regulated genes encoded proteins and transcription factors related to heat shock, and *HSP90* (*Csa3M183950.1*) was involved in the regulation of the hypersensitive response in the plant and pathogen interaction pathway. The expression of these genes might play a regulatory role in the process of embryogenesis.

The *BBM* gene was the first key gene to be isolated in the process of spore cell division; which was first expressed in zygotic embryogenesis and microspore embryogenesis (Pechan et al., 1991; Boutilier et al., 2002). Khanday et al (2019) have shown that BABYBOOM1 (BBM1) is a member of the family of transcription factors AP2 expressed in sperm cells and plays a key role in embryogenesis in rice (*oryza sativa*).

We found that two *BBM* genes (*Csa3M827310.1*, *Csa3M827320.1*) were up-regulated at the beginning of embryogenesis and were subsequently down-regulated. In addition, *AGL15* a member of the MADS family, was also considered a regulatory protein that acts at the start of cell division (Perry et al., 1999). We found that the *AGL* gene (*Csa3M258140.1*) was up-regulated in our study, indicating that all three of these genes play important roles in embryogenesis. We considered that *BBM, HSP90* and *AGL* might be the critical genes involved in the induction of embryogenesis by ovary culture in cucumber.

Microtubule organization genes play an important role in the embryogenesis of cucumber ovary culture

There is a similar process between gametophyte embryogenesis and zygotic embryogenesis, and the only difference is that the former occurs in sex cells, while the latter occurs in fertilized egg cells. The process of gametophyte embryogenesis development is similar to that of zygote differentiation into embryos. Microtubules play an important role in cells, between the polarized diffuse growth and the acquisition of novel cell fate, which would be also important for the zygote to initiate embryogenesis (Kimata Y et al., 2016). Electron microscope-based observations of

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carrot culture showed that the appearance of microtubules was accompanied with the formation of somatic embryos (Halperin et al., 1967). The different patterns of microtubule organization in the cells of the mature embryo sac reflect their structural adaptations for their future function (Huang et al., 1994).

In our study, after embryogenic potential was acquired, the different embryo shapes formed through active cell metabolism and rapid cell division. GO analysis showed that in different embryo periods continuously expressed genes participated in microtubule-based movement and cell or subcellular component processes, providing a good function of protein complex binding, microtubule binding, tetrapyrrole binding, tubulin binding and other microtubule activities; these genes mainly participated in protein processing of the endoplasmic reticulum and phenylpropanoid biosynthesis. We considered that the cellular reprogramming and morphological changes in embryos were controlled by microtubule organization genes.

Expression of main oxidation-reduction and metabolic process-related genes in cucumber ovary culture

Mature embryos are further cultured to form shoots, and at this stage, many biological processes are involved. GO analysis showed that differentially expressed genes participated in the oxidation-reduction process, a variety of metabolic processes, biosynthesis processes and the response to auxin, together with the molecular functions of oxidoreductase activity, binding capacity, catalytic activity, transporter activity, transcription factor activity and so on. Among them, the regulation of transcription, DNA template (GO: 0006355), the regulation of RNA biosynthetic processes (GO: 2001141), the regulation of RNA metabolic processes (GO: 0051252), DNA binding (GO: 0003677), transcription factor activity (GO: 0003700), and sequence-specific DNA binding (GO: 0043565) lay a foundation for cell division and differentiation. In addition, two processes, cellular response to auxin stimulus (GO: 0071365) and response to auxin (GO: 0009733), might promote the embryo to form shoots.

At the stage of shoot formation, a large number of transcription factors were expressed. The first constitutes transcription factors that contain a B3 domain (Stone et al., 2001). These transcription factors encode regulatory proteins involved in the embryonic development process, maintaining embryo development during late embryonic development (Boutilier et al., 2002; Riechmann and Meyerowitz, 1998). In our study, a total of 6 transcription factors (*Csa2M359980.1*, *Csa2M292240.1*, *Csa5M608380.1*, *Csa6M489980.1*, *Csa6M489940.1* and *CsaUNG031640*) that

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contain a B3 domain, were up-regulated. The second was the *AIL* gene from the AP2/ERF family, which is involved in key developmental processes throughout the whole plant life cycle. Some genes in the *AP2/ERF* gene family are expressed in many tissues and participate in many plant developmental processes, such as embryogenesis and shoot development (Boutilier et al., 2002; Riechmann and Meyerowitz, 1998). In addition, we found that the expression levels of 9 genes (*Csa1M423190.1*, *Csa3M114480.1*, *Csa3M652380.1*, *Csa3M114470.1*, *Csa4M644740.1*, *Csa5M175970.1*, *Csa5M608380.1*, *Csa6M496390.1*, and *CsaUNG031640*) in the AP2/ERF family were up-regulated. Furthermore, *WUS* homologous domain proteins not only alter the cell fate of the shoot and flower meristem but also promote the development of somatic embryos into seedlings. The function of *WUS* proteins has no direct connection with the characteristics of the embryo but will alter the development state of the tissue by maintaining cells in an undifferentiated state in response to different stimuli (Mayer et al., 1998; Gallois et al., 2004). The ability to transform the vegetative growth phase to the embryonic stage by *WUS*, as well as to eventually form somatic cells, indicates that this homologous domain protein also plays an important role in embryo maturation in addition to its role in the development of the meristem (Palovaara and Hakman, 2008). In the shoot apical, *WUS* defines the organizing center and is critical for induction of stem cell fate in the cells overlying the organizing center meristem (Dai et al., 2017; Meng et al., 2017).

During this stage, two genes encoding *WUS* homologous domain proteins (*Csa6M301060.1*) and (*Csa6M505860.1*) were up-regulated. We considered that those homologous domain proteins might directly or indirectly regulate shoot formation.

Conclusion

Several studies have reported on the embryogenesis of ovary culture in cucumbers. However, the mechanism that drives the process from embryogenic acquisition to the formation of embryos and plant regeneration is not well understood. In this study, we explored embryogenesis mechanism of ovary culture in cucumber. Inducing cucumber embryogenesis could be divided into three stages: early embryogenesis, embryo maturation (from proembryos to cotyledon embryos) and shoot formation (Fig. 9a).

The early stage of embryogenesis is the turning point for the formation of embryos, which had experienced dedifferentiation and loss of photosynthetic capacity, requiring

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the provision of exogenous nutrients and carbon sources such as plant hormones and sucrose in the media. Some physical factors, such as temperature, light quality, photoperiod and the presence of specific hormones, could affect the ability of the embryo sac to adapt to these conditions and survive the developmental transition. These dynamic changes were helpful for cell physiology reprogramming, metabolic alterations, revival of cell division and differentiation, morphogenesis and so on. Therefore, the cells of the embryo sac began to divide by dedifferentiation and started to form cell clumps (Fig. 1h~k). Although there was no obvious change in appearance, the metabolism of some macromolecules in the cells changed markedly. A large number of hormone-related genes, cell protection-related genes, and some unique protein kinases were expressed at the stage of early embryo development. transcription factors (*Csa6M147590.1* and *Csa3M389850.1*), the reported embryogenesis-related genes *BBM*, *HSP90* and *AGL* were also actively expressed during this stage (Fig. 9b).

In the stage of embryo maturation, GO analysis showed continuously expressed genes participated in microtubule-based movement, movement of the cell or subcellular component processes, giving a good function of protein complex binding, microtubule binding, tetrapyrrole binding, tubulin binding and other microtubule activities, which are involved in protein processing in the endoplasmic reticulum and phenylpropanoid biosynthesis(Fig. 9b).

Shoot formation was regulated by 6 transcription factors that contain a B3 domain, total of 9 genes in the *AP2/ERF* family and 2 genes encoding *WUS* homologous domain proteins (Fig. 9b).

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Figures

Fig. 1 Embryogenic process of ovary culture in cucumber

a-f and **l** observation under stereoscopic microscope. **a** Selected fresh unpollinated ovaries (0 d, T0). **b** Ovules were treated with the high temperature of 33 °C for 2 d; **c** Embryos cultured for 10 d, note the ovule enlargement and embryo initiation. **d** Embryos cultured for 10 d, with an average of 6 embryoids in each slice of ovary; **e** Embryos cultured for 30 d, embryo maturation and cotyledon-embryo formation. **f** Cultured for 60 d, the embryo differentiated into shoot. Histological observations: the histological sections were stained with Delafield's hematoxylin. **g** Multicellular embryo sac before culturing, **h** One of the cells expands in the embryo sac, after 2 d of culture, **i** Cell mitosis occurred at 4 d of culture. **j** Cells continue to be divided into four cells at 6 d of culture. **k** A cell clumps structure is visible at 8 d of culture. **l** Globular-embryo switch into cotyledon shape-embryo. **G-e** Globular embryo, **H-se** Heart-shaped embryo, **T-se** Torpedo-shaped embryo, **C-se** Cotyledon-shaped embryo, **Es** Embryo sac. The solid white lines represent 2mm, and the black lines represent 200μm. Ovule was marked with a white arrow.

Fig. 2 Validation of differentially expressed genes and correlation between RNA-seq and qRT-PCR

qRT-PCR of 7 up-regulated genes involved in plant hormone signal transduction and 3 plant-pathogen interaction genes in whole ovary culture were analyzed. **a** Csa6M067360.1, **b** Csa7M452370.1, **c** Csa1M006300.1, **d** Csa5M223020.1, **e** Csa5M434550.1, **f** Csa5M623800.1, **g** Csa6M383530.1, **h** Csa3M389850.1, **i** Csa7M420160.1, **j** Csa1M006320.1. Close correlations (Person's r=0.53, 0.77, 0.57, 0.74, 0.79, 0.69, 0.81, 0.86, 0.62, 0.81) were observed between relative expression levels in RNA-seq and qRT-PCR, validating the RNA-Seq methodology described here for quantitative analysis of the cucumber transcriptome. Three independent experimental replicates were analyzed for each sample, and data were showed as mean ± SE (n = 3).

Fig. 3 The total number of up-regulated and down-regulated genes

Black colored bars represent up-regulated genes, and gray ones represent down-regulated genes.

Fig. 4 KEGG pathway enrichment analysis based on the differentially expressed genes in early embryo development

The longitudinal coordinates are the enriched KEGG Pathways, and the x-axis monitors the rich factor; the size of the dots in the graph represents the number of differentially expressed genes annotated to the mentioned pathway, and the color represents the significant P value of the pathway. The 20 most significant pathways are shown in the picture.

Fig. 5 The matrix graph of up-regulated expression genes in the plant hormone signal transduction pathway in six time points

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The absolute values of the relative expression of up-regulated genes were indicated by the depth of color. The x-axis of the matrix graph showed the six time points. The y-axis of the matrix graph showed the name of the up-regulated genes. The up-regulated genes were arrayed according to the hormonal signal transduction flows, which were classified into six groups: auxin, cytokinin, abscisic acid, ethylene, brassinosteroid, salicylic acid.

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Fig. 6 Venn diagram showed differentially expressed genes in the stage from embryogenesis to the mature stage

The DEG sets (T1 vs T2, T2 vs T3 and T3 vs T4) described in Fig. 3 were analyzed using the Venn method. The numbers marked in the diagram indicated the number of genes significantly expressed among the three DEGs sets.

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Fig. 7: KEGG pathway enrichment analysis based on the differentially expressed genes from embryogenesis to the mature stage

The longitudinal coordinates indicate the KEGG pathways; the x-axis represents the rich factor; and the size of the dots in the graph represented the number of differentially expressed genes that were annotated to the respective pathway; the color represented the significant P value of the pathway. The 20 most significant pathways are shown in the picture.

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Fig. 8 KEGG pathway enrichment analysis based on the differentially expressed genes at shoot formation stage

The longitudinal coordinates indicate the KEGG pathways; the x-axis represents the rich factor; the size of the dots in the graph represented the number of differentially expressed genes that were annotated to the respective pathway, and the color represented the significant P value of the pathway. The 20 most significant paths are shown in the picture.

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Fig. 9 Pattern diagram of embryogenesis and plant formation induced by cucumber ovary culture

a Embryogenesis and plant formation process; **b** Molecular events at various stages; **ES** embryo sac; **Ge** globular embryo; **H-se** heart-shaped embryo; **T-se** torpedo embryo; **C-se** cotyledon embryo; **RP** regenerated plant.

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Additional files

Table S1. Statistics of RNA-Seq alignment

Table S2. GO classification of common expressed genes in gynogenesis stage.

Table S3. GO classification of common expressed genes from embryogenesis to the mature stage.

Table S4. GO classification of common expressed genes in organogenesis stage.

Table S5. Primers for Real-time quantitative PCR

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